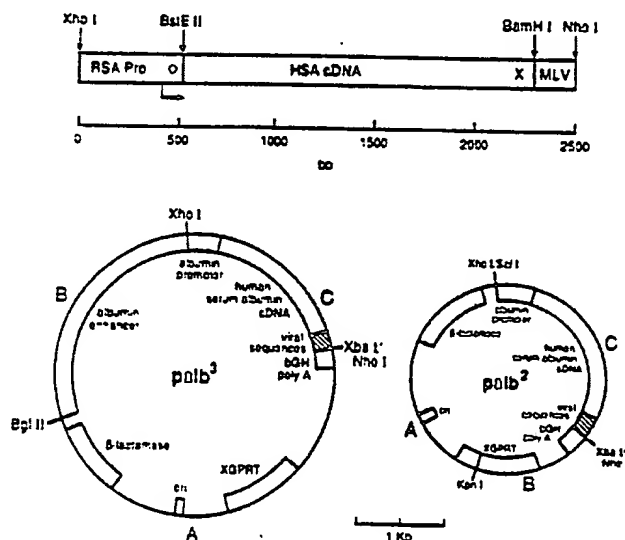




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(54) Title: TARGETED DELIVERY OF GENES ENCODING SECRETORY PROTEINS



## (57) Abstract

Molecular complexes for targeting a gene encoding a secretory protein to a specific cell *in vivo* and obtaining secretion of the protein by the targeted cell are disclosed. An expressible gene encoding a desired secretory protein is complexed to a conjugate of a cell-specific binding agent and a gene-binding agent. The cell-specific binding agent is specific for a cellular surface structure which mediates internalization of ligands by endocytosis. An example is the asialoglycoprotein receptor of hepatocytes. The gene-binding agent is a compound such as a polycation which stably complexes the gene under extracellular conditions and releases the gene under intracellular conditions so that it can function within a cell. The molecular complex is stable and soluble in physiological fluids and can be used in gene therapy to selectively transfect cells *in vivo* to provide for production and secretion of a desired secretory protein.

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-1-

TARGETED DELIVERY OF GENES  
ENCODING SECRETORY PROTEINS

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5 in part, by research grants from the United States  
government.

Background of the Invention

Many secreted proteins have been studied in a  
variety of cell types and all of them follow a similar  
10 pathway of secretion. The protein is synthesized in  
the cell cytosol by the process of translation which  
is performed by ribosomes located on the cytosolic  
side of the endoplasmic reticulum. The protein is  
then transported into the endoplasmic reticulum -  
15 Golgi apparatus for ultimate secretion from the cell.

The secretion of a protein is directed by a  
signal peptide which is usually located at the  
amino-terminus of the protein. This peptide is  
removed as the protein passes from the ribosome into  
20 the endoplasmic reticulum and therefore it does not  
appear in the mature, secreted protein.

Secretory proteins such as hormones or enzymes  
are involved in many biological processes. Severe  
abnormalities can result from the absence or  
25 insufficient secretion of such proteins. Methods for  
alleviating or correcting defects in the production of  
secretory proteins are needed.

-2-

Summary of the Invention

This invention pertains to a soluble molecular complex for targeting a gene encoding a secretory protein to a specific cell in vivo and obtaining  
5 secretion of the protein by the targeted cell. The molecular complex comprises an expressible gene encoding a desired secretory protein complexed to a carrier which is a conjugate of a cell-specific binding agent and a gene-binding agent. The  
10 cell-specific binding agent is specific for a cellular surface structure, typically a receptor, which mediates internalization of bound ligands by endocytosis, such as the asialoglycoprotein receptor of hepatocytes. The cell-specific binding agent can  
15 be a natural or synthetic ligand (for example, a protein, polypeptide, glycoprotein, etc.) or it can be an antibody, or an analogue thereof, which specifically binds a cellular surface structure which then mediates internalization of the bound complex.  
20 The gene-binding component of the conjugate is a compound such as a polycation which stably complexes the gene under extracellular conditions and releases the gene under intracellular conditions so that it can function within the cell.  
25 The complex of the gene and the carrier is stable and soluble in physiological fluids. It can be administered in vivo where it is selectively taken up by the target cell via the surface-structure-mediated endocytotic pathway. The incorporated gene  
30 is expressed and the gene-encoded product is processed and secreted by the transfected cell.

SUBSTITUTE SHEET

-3-

The soluble molecular complex of this invention can be used to specifically transfect cells in vivo to provide for expression and secretion of a desired protein. This selective transfection is useful for gene therapy and in other applications which require selective genetic alteration of cells to yield a secretable protein product. In gene therapy, a normal gene can be targeted to a specific cell to correct or alleviate an inherited or acquired abnormality involving a secretory protein, such as blood-coagulant deficiency, caused by a defect in a corresponding endogenous gene.

#### Brief Description Of The Figures

Figure 1 shows the structure of the plasmid vectors palb<sup>3</sup> and palb<sup>2</sup>, each of which contains a gene encoding the secretory protein albumin. Palb<sup>3</sup> contains the structural gene for human serum albumin driven by the rat albumin promoter and the mouse albumin enhancer regions. Palb<sup>2</sup> is a control vector which lacks the mouse albumin enhancer sequence which is necessary for high levels of expression of the albumin gene.

Figure 2 shows Southern blots which indicate the presence and abundance of plasmid DNA targeted by the method of this invention to liver cells of rats.

Figure 3 shows dot blots of hepatic RNA which indicate transcription of the vector-derived serum albumin gene by the liver cells.

Figure 4 shows RNase protection analysis which confirms the presence of vector-derived human serum albumin mRNA in the liver cells.

**SUBSTITUTE SHEET**

-4-

Figure 5 is a Western blot which confirms the presence of human serum albumin in rat serum.

Figure 6 shows levels of circulating human albumin in rat serum as a function of time after injection with palb<sup>3</sup> DNA complex and partial hepatectomy.

#### Detailed Description of the Invention

A soluble, targetable molecular complex is used to selectively deliver a gene encoding a secretory protein to a target cell or tissue in vivo. The molecular complex comprises the gene to be delivered complexed to a carrier made up of a binding agent specific for the target cell and a gene-binding agent. The complex is selectively taken up by the target cell and the gene product is expressed and secreted.

The gene, generally in the form of DNA, encodes the desired secretory protein (or glycoprotein). Typically, the gene comprises a structural gene encoding the desired protein in a form suitable for processing and secretion by the target cell. For example, the gene encodes appropriate signal sequences which provide for cellular secretion of the product. The signal sequence may be the natural sequence of the protein or exogenous sequences. The structural gene is linked to appropriate genetic regulatory elements required for expression of the gene product by the target cell. These include a promoter and optionally an enhancer element operable in the target cell. The gene can be contained in an expression vector such as a plasmid or a transposable genetic element along with the genetic regulatory elements necessary for expression of the gene and secretion of the gene-encoded product.

-5-

The carrier component of the complex is a conjugate of a cell-specific binding agent and a gene-binding agent. The cell-specific binding agent specifically binds a cellular surface structure which  
5 mediates its internalization by, for example, the process of endocytosis. The surface structure can be a protein, polypeptide, carbohydrate, lipid or combination thereof. It is typically a surface receptor which mediates endocytosis of a ligand.  
10 Thus, the binding agent can be a natural or synthetic ligand which binds the receptor. The ligand can be a protein, polypeptide, glycoprotein or glycopeptide which has functional groups that are exposed sufficiently to be recognized by the cell surface  
15 structure. It can also be a component of a biological organism such as a virus, cells (e.g., mammalian, bacterial, protozoan) or artificial carriers such as liposomes.

The binding agent can also be an antibody, or an  
20 analogue of an antibody such as a single chain antibody, which binds the cell surface structure.

Ligands useful in forming the carrier will vary according to the particular cell to be targeted. For targeting hepatocytes, glycoproteins having exposed  
25 terminal carbohydrate groups such as asialoglycoprotein (galactose-terminal) can be used, although other ligands such as polypeptide hormones may also be employed. Examples of asialoglycoproteins include asialoorosomucoid, asialofetuin and desialylated  
30 vesicular stomatitis virus. Such ligands can be formed by chemical or enzymatic desialylation of glycoproteins that possess terminal sialic acid and penultimate galactose residues. Alternatively,

-6-

asialoglycoprotein ligands can be formed by coupling galactose terminal carbohydrates such as lactose or arabinogalactan to non-galactose bearing proteins by reductive lactosamination.

5        For targeting the molecular complex to other cell surface receptors, other types of ligands can be used, such as mannose for macrophages (lymphoma), mannose-6-phosphate glycoproteins for fibroblasts (fibrosarcoma), intrinsic factor-vitamin B12 for  
10 enterocytes and insulin for fat cells.

Alternatively, the cell-specific binding agent can be a receptor or receptor-like molecule, such as an antibody which binds a ligand (e.g., antigen) on the cell surface. Such antibodies can be produced by  
15 standard procedures.

The gene-binding agent complexes the gene to be delivered. Complexation with the gene must be sufficiently stable in vivo to prevent significant uncoupling of the gene extracellularly prior to  
20 internalization by the target cell. However, the complex is cleavable under appropriate conditions within the cell so that the gene is released in functional form. For example, the complex can be labile in the acidic and enzyme rich environment of  
25 lysosomes. A noncovalent bond based on electrostatic attraction between the gene-binding agent and the expressible gene provides extracellular stability and is releasable under intracellular conditions.

Preferred gene-binding agents are polycations  
30 that bind negatively charged polynucleotides. These positively charged materials can bind noncovalently with the gene to form a soluble, targetable molecular complex which is stable extracellularly but



-7-

releasable intracellularly. Suitable polycations are polylysine, polyarginine, polyornithine, basic proteins such as histones, avidin, protamines and the like. A preferred polycation is polylysine (e.g.,  
5 ranging from 3,800 to 60,000 daltons). Other noncovalent bonds that can be used to releasably link the expressible gene include hydrogen bonding, hydrophobic bonding, electrostatic bonding alone or in combination such as, anti-polynucleotide anti-  
10 bodies bound to polynucleotide, and strepavidin or avidin binding to polynucleotide containing biotinylated nucleotides.

The carrier can be formed by chemically linking the cell-specific binding agent and the gene-binding  
15 agent. The linkage is typically covalent. A preferred linkage is a peptide bond. This can be formed with a water soluble carbodiimide as described by Jung, G. et al. Biochem. Biophys. Res. Commun.  
101:599-606 (1981). An alternative linkage is a  
20 disulfide bond.

The linkage reaction can be optimized for the particular cell-specific binding agent and gene-binding agent used to form the carrier. Reaction conditions can be designed to maximize  
25 linkage formation but to minimize the formation of aggregates of the carrier components. The optimal ratio of cell-specific binding agent to gene-binding agent can be determined empirically. When polycations are used, the molar ratio of the  
30 components will vary with the size of the polycation and the size of the gene. In general, this ratio ranges from about 10:1 to 1:1, preferably about 5:1. Uncoupled components and aggregates can be separated from the carrier by molecular sieve or ion exchange  
35 chromatography (e.g., Aquapore™ cation exchange, Rainan).

-8-

The gene encoding the secretory protein can be complexed to the carrier by a stepwise dialysis procedure. In a preferred method, for use with carriers made of polycations such as polylysine, the  
5 dialysis procedure begins with a 2M NaCl dialyzate and ends with a .15M NaCl solution. The gradually decreasing NaCl concentration results in binding of the gene to the carrier. In some instances, particularly when concentrations of the gene and  
10 carrier are low, dialysis may not be necessary; the gene and carrier are simply mixed and incubated.

The molecular complex can contain more than one copy of the same gene or one or more different genes. Preferably, the ratio of gene to the carrier  
15 is from about 1:5 to 5:1, preferably about 1:2.

The molecular complex of this invention can be administered parenterally. Preferably, it is injected intravenously. The complex is administered in solution in a physiologically acceptable vehicle.  
20

Cells can be transfected in vivo for transient expression and secretion of the gene product. For prolonged expression and secretion, the gene can be administered repeatedly. Alternatively, the transfected target cell can be stimulated to  
25 replicate by surgical or pharmacological means to prolong expression of the incorporated gene. See, for example, U.S. Patent Application Serial No. 588,013, filed September 25, 1990, the teachings of which are incorporated by reference herein.

30 The method of this invention can be used in gene therapy to selectively deliver a gene encoding a secretory protein to a target cell in vivo for expression and secretion of the gene-encoded product

-9-

by the cell. For example, a normal gene can be targeted to a specific cell to correct or alleviate a metabolic or genetic abnormality caused by an inherited or acquired defect in a corresponding  
5 endogenous gene.

The molecular complex of this invention is adaptable for delivery of a wide range of genes to a specific cell or tissue. Preferably, the complex is targeted to the liver by exploiting the hepatic  
10 asialoglycoprotein receptor system which allows for in vivo transfection of hepatocytes by the process of receptor-mediated endocytosis. The liver has the highest rate of protein synthesis per gram of tissue. Thus, the molecular complex of this  
15 invention can be used to specifically target the liver as a site for high efficiency production of a therapeutic secretory protein to treat hepatic abnormalities or abnormalities in other tissues.

The method of the invention can be used to treat  
20 inherited states of blood coagulant-deficiency. These include deficiencies in any of the clotting factors II-XIII. Factors V, VII, IX, X or XI are normally made in the liver. Factor VIII is normally made in endothelial cells and in liver parenchymal  
25 cells. In a preferred embodiment, the gene encoding the clotting factor is complexed to a conjugate of an asialoglycoprotein and a polycation. The resulting soluble complex is administered parenterally to target liver cells of the individual afflicted with  
30 the deficiency in amounts sufficient to selectively transfect the cells and to provide sufficient secretion of the factor to attain circulating levels for effective clotting activity.

-10-

This invention is illustrated further by the following Exemplification.

#### EXEMPLIFICATION

5

##### Example 1

An asialoglycoprotein-polycation conjugate consisting of asialoorosomucoid coupled to poly-L-lysine, was used to form a soluble DNA complex  
10 capable of specifically targeting hepatocytes via asialoglycoprotein receptors present on these cells. The DNA comprised a plasmid, palb<sup>3</sup>, containing the structural gene for human serum albumin driven by mouse albumin enhancer-rat albumin promoter elements.

15

##### Formation of the Molecular Complex

##### Animals

An animal model of a genetic metabolic disorder, the Nagase analbuminemic rat, was selected. This  
20 strain possesses a defect in splicing of mRNA of serum albumin resulting in virtually undetectable levels of circulating serum albumin (Nagase, S. et al. Science 205:590-591 (1979); Shalaby, F. and Shafritz, D.A. Proc. Natl. Acad. Sci. (USA)  
25 87:2652-26756 (1990)). Male, 200-250 g, Nagase analbuminemic rats were kindly provided by Dr. Jayanta Roy Chowdhury (Albert Einstein College of Medicine, Bronx, New York) and maintained in light-dark cycles and fed ad lib.

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-11-

Expression Vectors Containing the Human Serum Albumin Gene

The structures of the relevant portions of palbHSA, palb<sup>3</sup> and palb<sup>2</sup> are shown in figure 1.

- 5 XGPRT, xanthine-guanine phosphoribosyltransferase; MLV, Moloney murine leukemia virus; RSAPro, rat albumin promoter; HSA cDNA, human serum albumin cDNA; solid circle, translational start site; x, translational termination site.
- 10 The plasmid, palb<sup>3</sup>, is a eukaryotic expression vector that expresses human serum albumin cDNA sequences driven by the rat albumin promoter and the mouse albumin enhancer regions (Figure 1). This vector was constructed in a single three-part
- 15 ligation with fragments that were cloned in a directional manner. Fragment A: an XbaI to BglII fragment (3.7 kb) of plasmid MTEV.JT, the relevant sequences of which were derived from a precursor described by Pfarr, D.S. et al. DNA 4:461-467 (1988),
- 20 contains a 231 bp fragment of genomic DNA spanning the polyadenylation signal of the bovine growth hormone gene,  $\beta$ -lactamase and the prokaryotic origin of replication from PUC 19, and a eukaryotic transcriptional unit expressing xanthine-guanine
- 25 phosphoribosyltransferase (XGPRT). Fragment B: sequences spanning an enhancer located 5' to the mouse albumin gene (-12 to -9 kb) were excised from a pBR322 subclone of a recombinant lambda phage isolated from a mouse genomic library. Gorin, M.B.
- 30 et al. J. Biol. Chem. 256:1954-1959 (1981). The enhancer elements were removed on an EcoRV to BglII fragment in which the EcoRV site was converted to an XhoI site with synthetic linkers. Fragment C was

-12-

removed from a previously undescribed retroviral vector, palbHSA, as an XhoI to NheI fragment (2405 bp) which contains the following sequences: genomic DNA of the rat albumin gene from the XbaI site at 5 nucleotide -443 (converted to an XhoI site) to the BstEII site at nucleotide +45 (Urano, Y. et al. J. Biol. Chem. 261:3244-3251 (1986)); cDNA sequences of human serum albumin from the BstEII site at nucleotide +50 to the HindIII site at nucleotide 10 +1787 (converted to a BamHI site) (Urano, et al., supra) and 3' flanking sequences of the Moloney murine leukemia virus from the ClaI site at nucleotide 7674 (converted to a BamHI site) to the NheI site at nucleotide 7846 (Van Beveren, C., 15 Coffin, J., and Hughes, S. in RNA Tumor Viruses, Weiss, R., Teich, N., Varmus, H., and Coffin, J., eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 2nd ed. pp. 766-783 (1985)).

A control vector, palb<sup>2</sup>, lacking the albumin 20 enhancer was constructed (Figure 1) by a single three-part ligation as described above. Fragment A: an XbaI to KpnI fragment of plasmid MTEV.JT (2876 bp) containing the  $\beta$ -lactamase gene and the prokaryotic origin of replication from PUC 19 and a portion of a 25 eukaryotic transcriptional unit expressing XGPRT. Fragment B: a KpnI to SalI fragment of plasmid MTEV.JT (780 bp) containing the rest of the XGPRT transcriptional unit. Fragment C: an XhoI to NheI fragment (2405 bp) of palbHSA described above. 30 Because the enhancer regions are required for high level expression by the albumin promoter (Pinckert, C.A. et al. Genes and Development 1:268-276 (1987)) the palb<sup>2</sup> plasmid served to control the nonspecific effects of plasmid DNA.

-13-

The vectors were cloned in *E. coli* and purified as described previously (Birnboim, H.C., and Doly, J. Nucleic Acids Res. 7:1513-1518 (1979)). Purity was checked by electrophoresis through agarose gels stained with ethidium bromide (Maniatis, T., Fritsch, E.F., and Sambrook, G. in Molecular Cloning, A Laboratory Manual, Fritsch, E.G. and Maniatis, T., eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY pp. 150-161 (1982)).

10

#### The Targetable DNA Carrier

Asialoorosomucoid, prepared from pooled human serum (Wu, G.Y. and Wu, C.H. J. Biol. Chem. 263:14621-14624 (1988); Whitehead, D.H. and Sammons, H.G. Biochim. Biophys. Acta 124:209-211 (1966)), was coupled to poly-L-lysine (Sigma Chemical Co., St. Louis, MO), Mr = 3,800, as described previously using a water soluble carbodiimide (Jung, G. et al. Biochem. Biophys. Res. Commun. 101:599-606 (1981)).

In brief, asialoorosomucoid was treated with a 7-fold molar excess of poly-L-lysine at pH 7.4 using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Pierce Chemical Co., Rockford, IL) present in a 154-fold molar excess over poly-L-lysine. After 24 hrs, the conjugate product was purified by gel filtration chromatography and titrated with plasmid DNA using a gel retardation assay as described previously (Wu, G.Y. and Wu, C.H. J. Biol. Chem. 262:4429-4432 (1987)). The optimal ratio of conjugate to DNA for palb<sup>3</sup> was determined to be 2.5:1, and for palb<sup>2</sup>, 2.0:1. These ratios were used for all subsequent experiments. The complexed DNA was filtered through 0.45  $\mu$  membranes (Millipore Co., Bedford, MA) prior to injection.

35

-14-

#### Targeted Gene Delivery

Groups of rats, 2 each, were anesthetized with ketamine-xylazine and then injected intravenously via a tail vein with complexed palb<sup>3</sup> DNA, palb<sup>2</sup> DNA, 500 µg/ml in sterile saline, or saline alone. Fifteen minutes later, the rats were subjected to 66% partial hepatectomy (Wayforth, H.B., in Experimental and Surgical Techniques in the Rat, Academic Press, NY (1980)). At various intervals, blood was drawn, rats were killed, and livers removed and homogenized. DNA was isolated by phenol-chloroform extraction (Blin, N. and Stafford, D.W. Nucl. Acids. Res. 3:2303-2308 (1976)).

#### 15 Analysis of Targeted DNA

The quantity and state of human albumin DNA sequences were determined by Southern blot analysis (Southern, E.M. J. Mol. Biol. 98:503-517 (1975)). Liver DNA was isolated two weeks after injection with targeted DNA. Total cellular DNA was isolated and treated with BamHI, XhoI or NruI. Bands were detected by hybridization with <sup>32</sup>P-labeled probes derived from: 1) plasmid MTEV.JT, a 2307 bp EcoRI to BamHI fragment spanning the β-lactamase gene, or 2) the 3'-region of human serum albumin cDNA (1083 bp, BglII to BamHI fragment).

Figure 2 shows representative autoradiographs of DNA blots of liver DNA from Nagase analbuminemic rats 2 weeks after injection with targeted palb<sup>3</sup> DNA followed by partial hepatectomy. BamHI DNA from untransfected Nagase rat liver (10 µg) was supplemented with palb<sup>3</sup> plasmid DNA as follows: lane "0" contains no plasmid; lane "1C" contains 1 copy



-15-

(7.5 pg plasmid), lane "10C" contains 10 copies (75 pg plasmid), and lane "100C" contains 100 copies of plasmid/diploid genome (750 pg plasmid). XhoI and NruI DNA from untransfected Nagase rat liver (10 µg) was analyzed alone, lane "0", or in the presence of 50 copies of plasmid/diploid genome (375 pg plasmid), lane "50C". DNA from liver harvested 2 weeks after injection of complexed palb<sup>3</sup> DNA was analyzed in lane "palb<sup>3</sup>". BamHI and XhoI digested DNA blots were hybridized with an albumin cDNA probe; NruI digests were hybridized with the non-albumin-containing plasmid probe MTEV.JT, a 2307 bp EcoRI to BamHI fragment spanning the β-lactamase gene. Molecular size standards are indicated in kilobases along the right borders. (NC = nicked circular, L = linear, and SC = supercoiled DNA).

Restriction of total cellular DNA with BamHI releases the human albumin gene insert from the palb<sup>3</sup> plasmid on a 2100 bp fragment. As expected from the increasing amount of standard palb<sup>3</sup> added, lanes "1C", "10C" and "100C", show a proportional increase in hybridization of the band at approximately 2.1 kb, the size of the insert (left gel Figure 2). Another band found at approximately 9 kb, likely due to cross-hybridization to endogenous rat sequences (because it was also present in samples from untreated rats as shown in lane "0"), was used as an internal standard for the amount of cellular DNA present in each sample. No band corresponding in size to the insert was found in DNA from untreated rats, lane "0". However, rats treated with palb<sup>3</sup>, lane "palb<sup>3</sup>", showed a strong signal at the position expected for the insert, which upon quantitation

-16-

revealed an average copy number of 1000 copies of the plasmid/diploid genome. Bands larger than the albumin insert were not detected, indicating that no significant rearrangements of the albumin structural gene had occurred.

To characterize the molecular state of the plasmid DNA in palb<sup>3</sup>-treated liver samples 2 weeks post-injection and partial hepatectomy, total cellular DNA digested with XhoI, which has a single cutting site in the plasmid, and hybridized with the albumin cDNA probe. Figure 2, middle gel, lane "palb<sup>3</sup>", shows that digestion of palb<sup>3</sup>-treated liver DNA produced a band that corresponded in size to linearized plasmid. Hybridization to some endogenous rat sequences was also seen in the form of bands greater than 14 kb in size.

To confirm that DNA bands corresponding in size to plasmid were indeed of plasmid origin, total cellular DNA from livers from the palb<sup>3</sup>-treated rats were digested with NruI which lacks any restriction sites in the plasmid. Samples were probed with a fragment of the plasmid MTEV.JT, spanning the  $\beta$ -lactamase gene but lacking any albumin sequences. This showed two predominant bands corresponding to nicked circular and supercoiled forms of the plasmid. A small band was also seen, corresponding to linearized plasmid. These data indicate that the overwhelmingly predominant portion of retained DNA in liver in these experiments existed as unintegrated circular plasmid DNA. However, because of the presence of hybridizable high molecular weight DNA, the possibility of integration of some plasmid DNA into the host genome cannot be excluded. Rats treated with the enhancerless palb<sup>2</sup> plasmid showed similar patterns.

SUBSTITUTE SHEET

-17-

Analysis of Human Albumin mRNA: RNA Dot-Blots

In order to determine whether the targeted, complexed DNA was transcribed, analbuminemic rat livers were assayed by dot blots for the presence of human serum albumin mRNA two weeks after injection and partial hepatectomy. A representative dot blot of RNA extracted from Nagase analbuminemic rat livers from animals 2 weeks after treatment with targeted plasmid DNA or controls followed by partial hepatectomy.

Total RNA was extracted from liver tissue by the method of Chomczynski *et al.* (Chomczynski, P. and Sacchi, N. *Anal. Biochem.* 162:156-159 (1987)). Serial (1:2) dilutions of RNA starting at 30 µg with or without pretreatment with DNase-free RNase were applied onto a nitrocellulose filter and hybridized to a <sup>32</sup>P-labeled 19-mer synthetic cDNA specific for a human albumin sequence (complementary to sequences of albumin message corresponding to the 695-715 base pair region of human albumin cDNA). Sambrook, J., Fritsch, E.F. and Maniatis, T., eds. Cold Spring Harbor, New York pp. 7.35-7.55 (1989). Row 1, analbuminemic rats treated with saline; row 2, analbuminemic rats treated with palb<sup>2</sup> plasmid DNA as a targetable complex; row 3, analbuminemic rats treated with palb<sup>3</sup> as a targetable complex; row 4, same as row 3 except that the sample was digested with DNase-free RNase prior to hybridization; row 5, RNA from normal Sprague-Dawley rats. NAR, Nagase analbuminemic rats.

-18-

As shown in Figure 3, total RNA from livers of rats that received saline alone, top row; as well as rats that received the enhancerless control plasmid, *palb*<sup>2</sup>, second row, did not hybridize with the human albumin specific cDNA probe. However, the third row shows that RNA from rats that received the *palb*<sup>3</sup> did produce a strong signal. The fourth row (in which a sample from row 3 was digested with DNase-free RNase prior to hybridization) shows that DNase-free RNase completely abolished the hybridization seen previously in row 3, supporting the conclusion that the signal was due to the presence of RNA. The last row shows that RNA from liver of a normal untreated Sprague-Dawley rat did not hybridize with the probe, indicating that the signal detected in row 3 was not due to hybridization to endogenous rat sequences.

Analysis of Human Albumin mRNA: RNase Protection Assays

Further evidence for the presence of vector-derived human serum albumin mRNA in liver tissue was provided by RNase protection analysis using a vector-specific RNA probe followed by partial hepatectomy.

RNA was extracted from liver tissue and analyzed by RNase protection assays (Melton, D.A. *et al.* Nucleic Acids Res. 12:7035-7056 (1984)) using a vector-specific probe. The RNA probe, 3Z-env, complementary to Moloney retrovirus-derived sequences in the 3' untranslated region of the recombinant human albumin transcript was synthesized in vitro as

-19-

described previously (Wilson, J.M. *et al.* Proc. Natl. Acad. Sci. 87:8437-8441 (1990)) by cloning this region between the BamHI and XbaI sites of pGEM-3Z(f+), and labeling with  $^{32}\text{P}$ .

5 RNA from a previously transfected NIH 3T3 cell line that expresses a transcript containing the vector-derived sequence, and RNA from the untransfected NIH 3T3 cells were used as positive and negative controls, respectively. Total cellular RNA  
10 from liver tissue was extracted as described above, and 100  $\mu\text{g}$  each were analyzed by RNase protection according to the method of Melton *et al.* (supra). Lane "3T3" contains RNA (200 ng) from an NIH 3T3 cell line that was made to express a transcript which  
15 possesses the vector-derived sequence. A 172 bp fragment that is resistant to RNase A was found at the expected location indicated by the arrow. Lane "palb<sup>2</sup>" contains RNA (100  $\mu\text{g}$ ) from analbuminemic rat liver harvested 2 weeks after transfection with  
20 palb<sup>2</sup>; and lane "palb<sup>3</sup>", RNA (100  $\mu\text{g}$ ) from analbuminemic rat liver harvested 2 weeks after transfection with palb<sup>3</sup>. Molecular size markers are present in the lane farthest to the right.

Hybridization of the probe to RNA from NIH 3T3  
25 cells made to express the transcript containing vector sequences (positive control cells), produced a band of the expected size, 172 bp (arrow) that was resistant to digestion with RNase A as shown in Figure 4, lane "3T3". Analysis of RNA from liver  
30 harvested 2 weeks after transfection of analbuminemic rats with palb<sup>3</sup> DNA complex followed by partial hepatectomy also resulted in a protected band of the expected size (172 bp). Some higher size bands were

-20-

also present, likely due to incomplete digestion of the hybrid with RNase. However, liver from analbuminemic rats harvested 2 weeks after transfection and partial hepatectomy using the same 5 molar quantities of complexed palb<sup>2</sup> DNA as in the palb<sup>3</sup> DNA experiments, Figure 4, lane "palb<sup>2</sup>" failed to generate any protected sequences under identical conditions. Similarly, RNA from untransfected NIH 3T3 cells, and untransfected Nagase analbuminemic rats did not produce protected sequences indicating that the observed 172 bp band obtained after palb<sup>3</sup> DNA transfection was not due to non-specific hybridization to other endogenous, non-vector-derived RNA sequences. Using RNase protection analysis with probes to endogenous rat albumin and recombinant human albumin on RNA, the level of human albumin mRNA in transfected analbuminemic rat liver was estimated to be between 0.01% and 0.1% of rat albumin mRNA in normal rats (data not shown).

20

#### Assay for Circulating Human Serum Albumin

Identification and quantitation of human serum albumin was accomplished by Western blots (Burnette, W.N. Anal. Biochem. 112:195-203 (1981)), using an affinity-purified rabbit anti-human albumin antibody. Figure 5 is a representative Western blot of rat serum samples taken two weeks after treatment of analbuminemic rats with targeted palb<sup>3</sup> DNA followed by partial hepatectomy. Serum or standard albumins were applied on a polyacrylamide gel electrophoresis, then transferred to nitrocellulose and exposed to the specific rabbit anti-human albumin antibody. Subsequently the gels were incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase and developed by exposure to BCIP/NBT.

-21-

Specifically, 10 µg of human serum albumin, 10 µg rat serum albumin, and 4 µl each of serum from normal rats, untreated analbuminemic rats, and treated analbuminemic rats were applied onto a 10% SDS-polyacrylamide gel (Laemmli, U.K. Nature 227:680-685 (1970)) and run at 150 V for 4.5 hours. Human serum albumin, 20 µg, is shown in lane 1; standard rat serum albumin, 20 µg, lane 2; human albumin, 20 µg, in 4 µl untreated analbuminemic rat serum, lane 3; and 4 µl of serum from: untreated analbuminemic rats, lane 4; normal Sprague-Dawley rats, lane 5; serum from analbuminemic rats treated with palb<sup>3</sup> DNA complex, lane 6; analbuminemic rats treated with saline alone, lane 7; analbuminemic rats treated with palb<sup>2</sup> DNA complex, lane 8.

The gel was electrophoretically transferred onto nitrocellulose using a Trans-Blot cell (Bio-Rad), quenched with blotto (10% powdered non-fat milk in PBS), exposed to anti-human albumin antibody, and then incubated with anti-rabbit IgG conjugated to alkaline phosphatase. The filters were then washed, and developed with BCIP/NBT (Kirkegaard and Perry Lab. Inc.)

Figure 5, lanes 1-5 demonstrate the specificity of the anti-human serum albumin antibody for human albumin; a single band was detected in the blot of standard human albumin, whereas no staining was detected with an equal amount of standard rat serum albumin, lane 2. Albumin is known to bind a number of serum components. To determine whether binding of rat serum components could alter the electrophoretic mobility of human albumin, standard human albumin was mixed with serum from untreated analbuminemic rats.

-22-

Lane 3 shows that this had no significant effect as the migration position of human albumin remained unchanged. A band at approximately 130 kDa is likely due to the presence of albumin dimers.

5       The specificity of the anti-human albumin antibody was further demonstrated by the lack of any reaction to either normal rat serum, lane 4; or untreated analbuminemic rat serum, lane 5. However, analbuminemic rats that received the palb<sup>3</sup> DNA  
10 complex did produce a band corresponding in size to albumin. The level of this circulating human serum albumin was quantitated to be approximately 30 µg/ml, two weeks after injection, lane 6. Control animals that received saline alone, lane 7, or the palb<sup>2</sup>  
15 enhancerless plasmid, lane 8, did not produce detectable human albumin under identical conditions.

A time course of the appearance of human albumin in the circulation is shown in Figure 6. Rats were treated with palb<sup>3</sup> DNA complex followed by partial  
20 hepatectomy. At regular intervals, serum was obtained and levels of circulating human serum albumin determined by Western blots as described for Figure 4. Lanes 1-3 contain standard human albumin, 0.1, 1.0 and 10 µg. Lanes 4-11 contain 4 µl serum  
25 from treated rats 24 h, 48 h, 72 h, 96 h, 1 week, 2 weeks, 3 weeks, and 4 weeks after injection, respectively. Serum samples or standard human albumin were applied on a polyacrylamide gel electrophoresis, then transferred to nitrocellulose  
30 and exposed to a specific rabbit anti-human albumin antibody. Filters were washed and then incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase and developed by exposure to BCIP/NBT.



-23-

Serum from a representative analbuminemic rat treated with palb<sup>3</sup> DNA complex, lane 4, did not have detectable circulating albumin after 24 hours. However, human albumin was detectable in serum from 5 palb<sup>3</sup> DNA-treated analbuminemic rats by 48 hours, lane 5, at a level of approximately 0.05 µg/ml. The level of human albumin rose with time reaching a plateau of 34 µg/ml by the 2nd week, lane 8, and remained at this level without significant change 10 through the 4th week post-injection, lane 11. Using an ELISA method, no anti-human albumin antibodies were detected, at least through the 4th week after transfection (data not shown).

#### 15 Example 2

An asialoglycoprotein-polycation conjugate consisting of asialoorosmucoid coupled to poly-L-lysine, was used to form a soluble DNA complex capable of specifically targeting hepatocytes via 20 asialoglycoprotein receptors present on these cells. The DNA comprised a plasmid containing the gene for hepatitis B virus surface antigen.

#### Expression Vector Containing Gene Encoding Hepatitis 25 B Virus Surface Antigen

Plasmid pSVHBVs was obtained from Dr. T. Jake Liang (Massachusetts General Hospital, Boston, MA). The plasmid (approximately 3.6kbp) is a pUC derivative containing the SV40 origin of replication 30 and the open reading frame for hepatitis B surface antigen (as part of a 1984 bp insert) driven by the SV40 promoter. The plasmid was cloned and purified as described above.

-24-

The Targetable DNA Carrier

Asialoorosmucoid (ASOR) was prepared as described above. ASOR was coupled to poly-L-lysine (Sigma Chemical Co., St. Louis, MO) Mr = 59,000 (7:1 molar ratio) via disulfide bonds using N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) to form the labeled conjugate. ASOR was also coupled to poly-L-lysine Mr = 41,100 (1:1 molar ratio) at pH 7.4 using 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (Pierce Chemical Co., Rockford, IL).

The conjugates were purified by cation exchange chromatography using a high pressure liquid chromatographic system (Rainan) employing an Aquapore C-300 column (Rainan) and stepwise elution with 0.1 M sodium acetate pH 5.0, 2.5, 2.25 and 2.0. The second peak eluted from the column as detected by U.V. absorption at 230 nm was determined as the optimal conjugate (Jung, G. et al. Biochem Biophys. Res. Commun. 101:599-606 (1981)).

The optimal proportion of DNA to mix with the conjugate to form a soluble complex was determined using gel retardation assay described above. Samples containing equal amounts of DNA in .15 M NaCl were mixed with increasing amounts of the conjugate in .15 M NaCl to determine the conjugate to DNA molar ratio which completely retards DNA migration in the gel. The amount of conjugate needed to bind 50-75% of the DNA was calculated and used to form the molecular complex (in order to ensure solubility of the complex). To form the soluble molecular complex, the conjugate solution was added very slowly to the DNA solution by a peristaltic pump at a speed of 0.1 ml/min with constant mixing. An aliquot was taken

-25-

and absorbance at  $A_{260\text{nm}}$  was determined to monitor the amount of DNA. Another aliquot was taken and run on an agarose gel to verify the formation of complex. The solution containing the complex was 5 filtered through a  $0.45\ \mu$  membrane filter and washed with saline. Aliquots were taken for testing as above.

#### Targeted Gene Delivery

10        Groups of 150 g female rats (Sprague-Dawley), 2 each were anesthetized with hetamine-xylazine and then injected very slowly intravenously via the tail vein. Rats in one group received the conjugate prepared with poly-L-lysine  $M_r = 59,000$  using SPDP 15 coupling and complexed with 5 mg DNA. The other group of rats received the conjugate prepared with poly-L-lysine  $M_r = 41,100$  using carbodiimide coupling and complexed with 1.4 mg DNA. At 24 hour intervals, the rats were bled and serum was be obtained for 20 assay of hepatitis-B virus surface antigen. (Auszyme Monoclonal, EIA Kit for detection of HBV - Abbott). The resultant solution color change was measured at  $A_{492\text{nm}}$  for 200  $\mu$ l of serum. The results are shown in Table 1.

25

-26-

Table 1

Results are given as optical density units  
at A<sub>492nm</sub> for 200 µl serum

5

Time (Days)

	0	1	2	3	4	6	7	8	14	30	60
Rat											
1	.012	.024	.261	.33	.23						
2	.011	.048	.137	.28	.25	.22	.10	.09	.175	.33	.15
3	.016	.26	.22	.08							
4	.018	.22	.24	.16							

15 The expression HBV surface antigen detected for the rats that received the soluble molecular complex consisting of the conjugate prepared via SPDP coupling and 5 mg DNA (rats #1 & #2) persisted for at least 4 days and increased consistently reaching a maximum of  
20 .33. The expression detected for the rats that received the soluble molecular complex consisting of the conjugate prepared via carbodiimide coupling and 1.4 mg DNA (rats 13 & #4) also persisted for at least 3 days and increased consistently reaching a maximum  
25 of approximately .25.

#### Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine  
30 experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

-27-

Claims

1. A soluble molecular complex for targeting a gene encoding a secretory protein to a specific cell, the  
5 complex comprising an expressible gene encoding the secretory protein complexed with a carrier comprising a cell-specific binding agent and a gene-binding agent which complexes the gene under extracellular condition and releases the gene under intracellular  
10 condition as an expressible molecule.
2. A soluble molecular complex of claim 1, wherein the expressible gene is DNA.
- 15 3. A soluble molecular complex of claim 1, wherein the expressible gene encodes albumin.
4. A soluble molecular complex of claim 1, wherein the expressible gene encodes a blood coagulation  
20 factor.
5. A soluble molecular complex of claim 4, wherein the blood coagulation factor is selected from the group consisting of factor V, VII, VIII, IX, X or XI.  
25
6. A soluble molecular complex of claim 1, wherein the gene-binding agent is a polycation.
7. A soluble molecular complex of claim 6, wherein  
30 the polycation is polylysine.
8. A soluble molecular complex of claim 1, wherein the cell-specific binding agent binds a surface receptor of the cell which surface receptor mediates  
35 endocytosis.

-28-

9. A soluble molecular complex of claim 8, wherein the cell-specific binding agent is a ligand for an asialoglycoprotein receptor.
- 5 10. A soluble molecular complex of claim 9, wherein the ligand is an asialoglycoprotein and the targeted cell is a hepatocyte.
11. A soluble molecular complex of claim 1, wherein  
10 the expressible gene is complexed with the gene-binding agent by a noncovalent bond.
12. A soluble molecular complex of claim 1, wherein the cell-specific binding agent is linked to the  
15 gene-binding agent by a covalent bond.
13. A soluble molecular complex of claim 1, wherein the expressible gene is complexed with the gene-binding agent so that the gene is released in  
20 functional form under intracellular conditions.
14. A pharmaceutical composition comprising a solution of the molecular complex of claim 1 and physiologically acceptable vehicle.
- 25 15. A soluble molecular complex for targeting a gene encoding a secretory protein to a hepatocyte, the complex comprising an expressible gene encoding the secretory protein complexed with a carrier comprising  
30 a ligand for the asialoglycoprotein receptor and a polycation which complexes the gene under extracellular condition and releases the gene under intracellular condition as an expressible molecule.
- 35 16. A soluble molecular complex of claim 15, wherein the expressible gene encodes albumin.

-29-

17. A soluble molecular complex of claim 15, wherein the expressible gene encodes a blood coagulation factor.
- 5 18. A soluble molecular complex of claim 17, wherein the blood coagulation factor is selected from the group consisting of factor V, VII, VIII, IX, X or XI.
- 10 19. A soluble molecular complex of claim 15, wherein the polycation is polylysine.
20. A soluble molecular complex of claim 15, wherein the gene is contained in an expression vector along with genetic regulatory elements necessary for  
15 expression of the gene and secretion of a gene-encoded product by the hepatocyte.
21. A soluble molecular complex of claim 20, wherein the expression vector is a plasmid or viral DNA.  
20
22. A soluble molecular complex for targeting a gene encoding factor VIII protein to a hepatocyte, the complex comprising an expressible gene encoding the factor VIII protein complexed with a carrier  
25 comprising a ligand for the asialoglycoprotein receptor and a polycation which complexes the gene under extracellular condition and releases the gene under intracellular condition as an expressible molecule.
- 30 23. A soluble molecular complex of claim 22, wherein the polycation is polylysine.

-30-

24. A soluble molecular complex for targeting a gene encoding factor IX protein to a hepatocyte, the complex comprising an expressible gene encoding the factor IX protein complexed with a carrier comprising  
5 a ligand for the asialoglycoprotein receptor and a polycation which complexes the gene under extracellular condition and releases the gene under intracellular condition as an expressible molecule.
- 10 25. A soluble molecular complex of claim 24, wherein the polycation is polylysine.
26. A method of delivering an expressible gene encoding a secretory protein to a specific cell of an  
15 organism for expression and secretion of the gene-encoded product by the cell, comprising administering to the organism a soluble molecular complex comprising the expressible gene encoding the secretory protein complexed with a carrier comprising  
20 a cell-specific binding agent and a gene-binding agent which complexes the gene under extracellular condition and releases the gene under intracellular condition as an expressible molecule.
- 25 27. A method of claim 26, wherein the expressible gene is DNA.
28. A method of claim 26, wherein the expressible gene encodes albumin.
- 30 29. A method of claim 26, wherein the expressible gene encodes a blood coagulation factor.
30. A method of claim 29, wherein the blood  
35 coagulation factor is selected from the group consisting of factor V, VII, VIII, IX, X and XI.



-31-

31. A method of claim 26, wherein the gene-binding agent is a polycation.

32. A method of claim 31, wherein the polycation is  
5 polylysine.

33. A method of claim 26, wherein the cell-specific binding agent binds a surface receptor of the cell which surface receptor mediates endocytosis.

10

34. A method of claim 33, wherein the cell-specific binding agent is a ligand for an asialoglycoprotein receptor.

15 35. A method of claim 34, wherein the ligand is an asialoglycoprotein and the targeted cell is a hepatocyte.

36. A method of claim 26, wherein the molecular  
20 complex is administered intravenously.

37. A method of selectively transfecting hepatocytes in vivo with a gene encoding a secretory protein, comprising intravenously injecting a pharmaceutically  
25 acceptable solution of a molecular complex comprising an expressible gene encoding the secretory protein complexed with a carrier comprising a ligand for the asialoglycoprotein receptor and a polycation which complexes the gene under extracellular condition and  
30 releases the gene under intracellular condition as an expressible molecule.

38. A method of claim 37, wherein the hepatocytes are transfected to correct or alleviate an inherited  
35 or acquired abnormality in an organism.

-32-

39. A method of claim 38, wherein the expressible gene encodes albumin.

40. A method of claim 38, wherein the expressible  
5 gene encodes a blood coagulation factor.

41. A method of claim 40, wherein the blood  
coagulation factor is selected from the group  
consisting of factor V, VII, VIII, IX, X and XI.

10

42. A method of claim 37, wherein the polycation is  
polylysine.

43. A method of claim 37, wherein the expressible  
15 gene is contained in an expression vector along with  
genetic regulatory elements necessary for expression  
of the gene and secretion of the gene-encoded product  
by the hepatocyte.

20 44. A method of claim 43, wherein the expression  
vector is a plasmid or a viral genome.

45. A soluble molecular complex of claim 1, wherein  
the ratio of carrier to gene ranges from 1:5 to 5:1.

25

46. A soluble molecular complex of claim 15, wherein  
the ratio of carrier to gene ranges from 1:5 to 5:1.

47. A method of claim 26, wherein the ratio of  
30 carrier to gene in the molecular complex is 1:5 to  
5:1.

48. A method of claim 37, wherein the ratio of  
carrier to gene in the molecular complex is 1:5 to  
35 5:1.

1/6

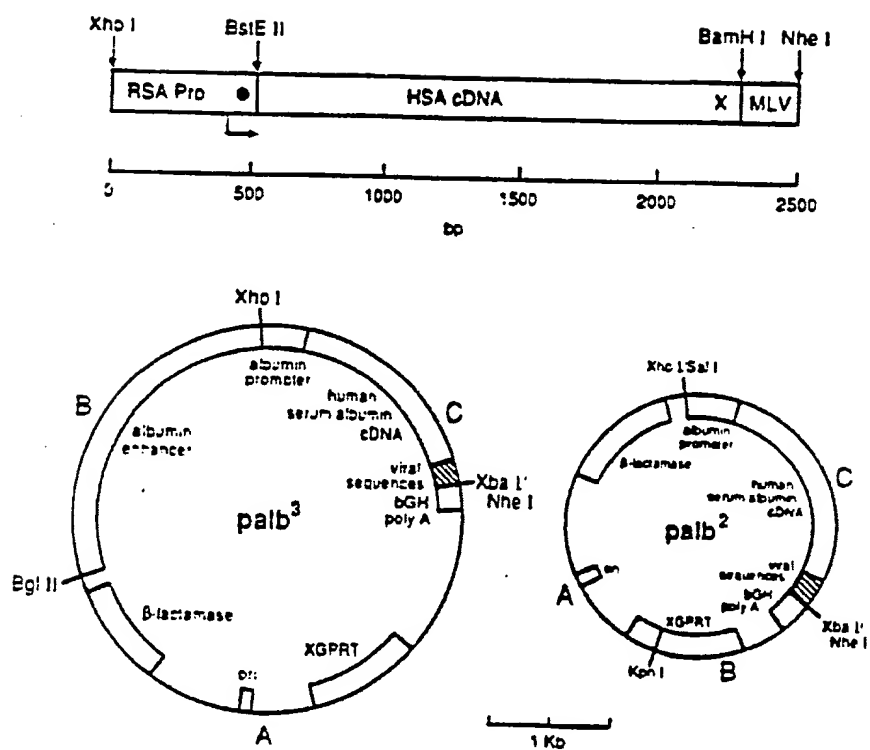


Figure 1

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2 / 6

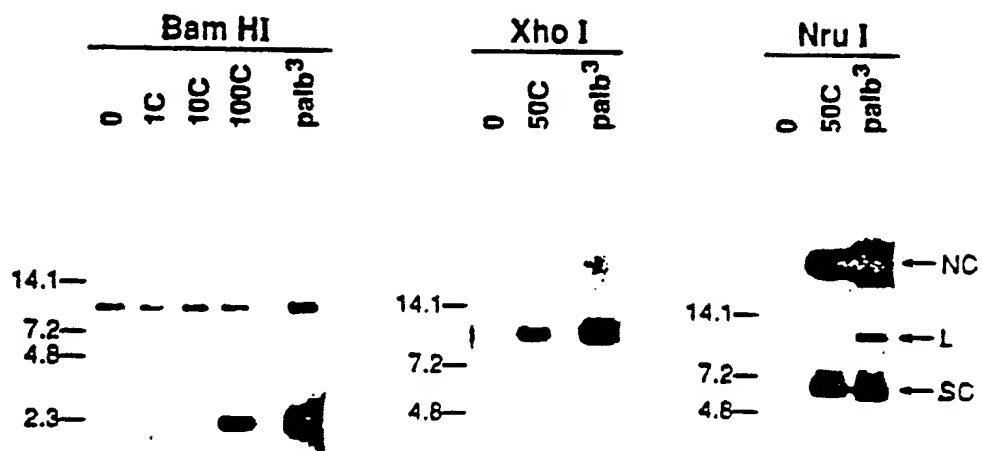


Figure 2

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3 / 6

NAR	Saline	
NAR	palb <sup>2</sup>	
NAR	palb <sup>3</sup>	
NAR	palb <sup>3</sup> + RNase	
Normal		

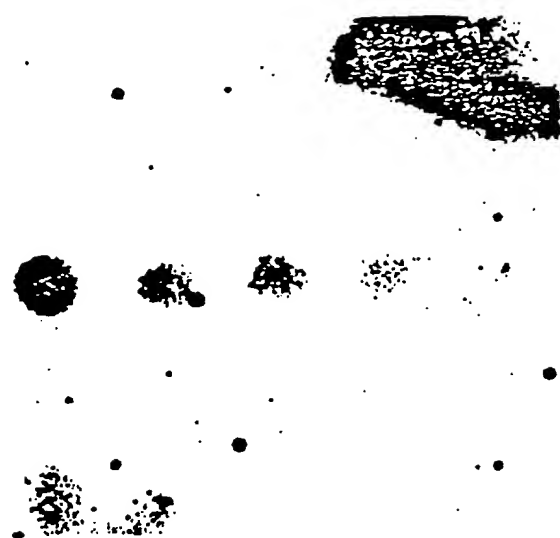


Figure 3

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4 / 6

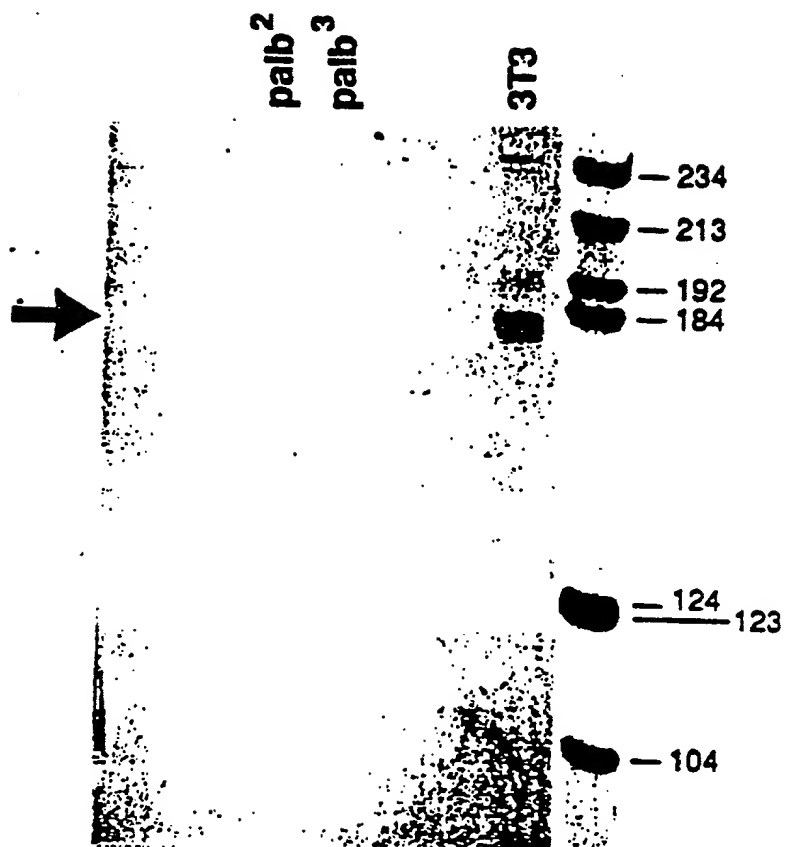


Figure 4

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5 / 6

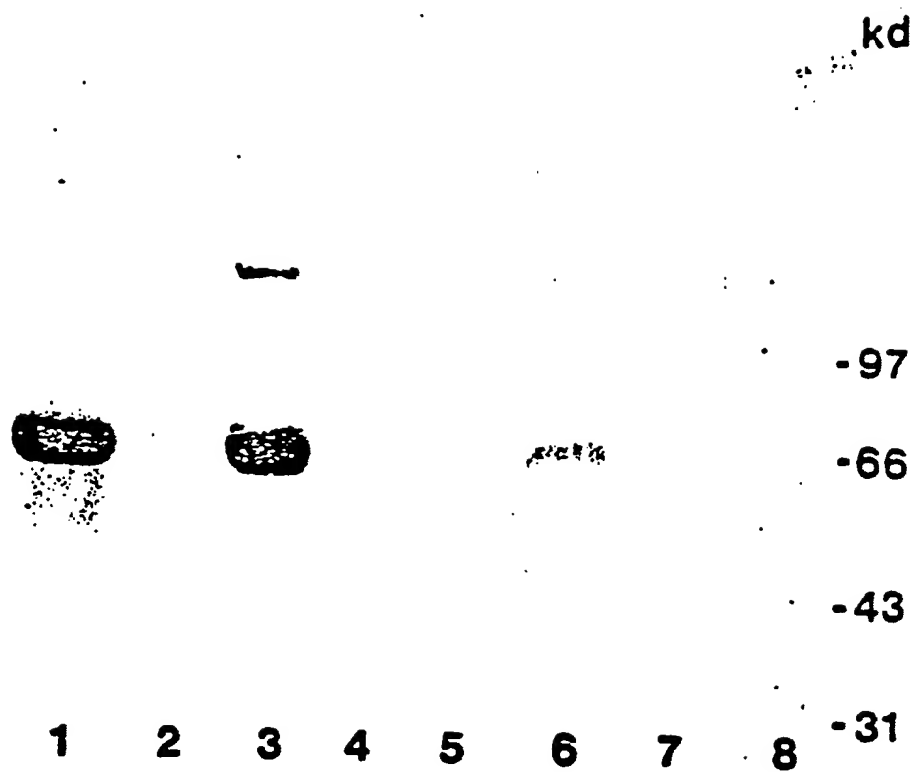


Figure 5

6 / 6

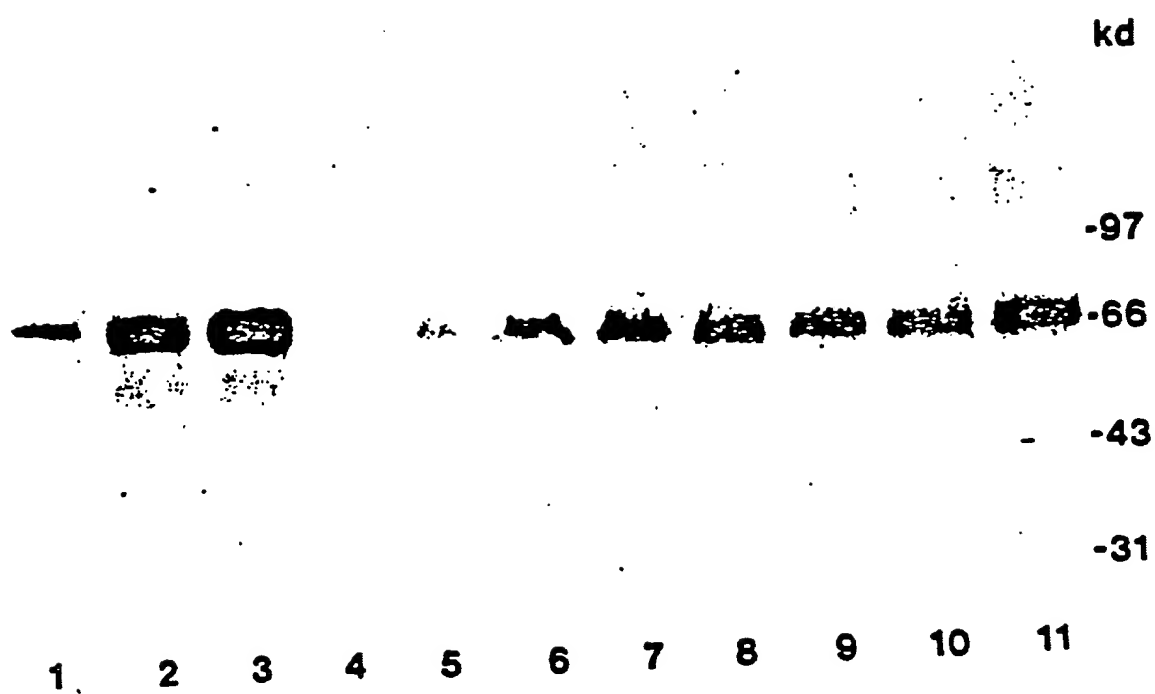


Figure 6


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## INTERNATIONAL SEARCH REPORT

PCT/US 92/04565

International Application No

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 A61K47/48		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	A61K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
A	JOURNAL OF BIOLOGICAL CHEMISTRY vol. 264, no. 29, 15 October 1989, BALTIMORE, USA pages 16985 - 16987 WU ET AL 'TARGETING GENES: DELIVERY AND PERSISTENT EXPRESSION OF A FOREIGN GENE DRIVEN BY MAMMALIAN REGULATORY ELEMENTS IN VIVO' see the whole document ---	1-48
A	JOURNAL OF BIOLOGICAL CHEMISTRY vol. 264, no. 21, 25 July 1989, BALTIMORE, USA pages 12126 - 12129 KANEDA ET AL 'INTRODUCTION AND EXPRESSION OF THE HUMAN INSULIN GENE IN ADULT RAT LIVER' see the whole document ---	1-48
-/--		
<p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
29 OCTOBER 1992	24. 11. 92	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	SITCH W.D.C. 	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		Relevant to Claim No.
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	
A	JOURNAL OF BIOLOGICAL CHEMISTRY vol. 266, no. 6, 25 February 1991, BALTIMORE, USA pages 3361 - 3364 KATO ET AL 'EXPRESSION OF HEPATITIS B VIRUS SURFACE ANTIGEN IN ADULT RAT LIVER' see the whole document ---	1-48
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA vol. 87, April 1990, WASHINGTON D.C., USA pages 2652 - 2656 SHALABY ET AL 'EXON SKIPPING DURING SPLICING OF ALBUMIN MRNA PRECURSORS IN NAGASE ANALBUMINEMIC RATS' cited in the application see P.2652, abstract ---	1-48
A	WO, A, 9 012 096 (PURDUE RESEARCH FOUNDATION) 18 October 1990 see page 1, line 25 - page 7, line 4 ---	1-48
P, X	JOURNAL OF BIOLOGICAL CHEMISTRY vol. 266, no. 22, 5 August 1991, BALTIMORE, USA pages 14338 - 14342 WU ET AL 'RECEPTOR-MEDIATED GENE DELIVERY IN VIVO' see the whole document -----	1-3, 6-16, 19-21, 26-28, 31-39, 42-48

US 9204565  
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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82